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DOI: https://doi.org/10.1007/s00427-004-0464-7

Dev Genes Evol (2005) 215: 165–176
DOI 10.1007/s00427-004-0464-7

ORIGINAL ARTICLE

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The *transformer2* gene in *Musca domestica* is required for selecting and maintaining the female pathway of development

Received: 4 November 2004 / Accepted: 8 December 2004 / Published online: 21 January 2005

Abstract We present the isolation and functional analysis of a *transformer2* homologue *Mdtra2* in the housefly *Musca domestica*. Compromising the activity of this gene by injecting dsRNA into embryos causes complete sex reversal of genotypically female individuals into fertile males, revealing an essential function of *Mdtra2* in female development of the housefly. *Mdtra2* is required for female-specific splicing of *Musca doublesex* (*Mddsx*) which structurally and functionally corresponds to *Drosophila dsx*, the bottom-most regulator in the sex-determining pathway. Since *Mdtra2* is expressed in males and females, we propose that *Mdtra2* serves as an essential co-factor of *F*, the key sex-determining switch upstream of *Mddsx*. We also provide evidence that *Mdtra2* acts upstream as a positive regulator of *F* supporting genetic data which suggest that *F* relies on an autocatalytic activity to select and maintain the female path of development. We further show that repression of male courtship behavior by *F* requires *Mdtra2*. This function of *F* and *Mdtra2* appears not to be mediated by *Mddsx*, suggesting that bifurcation of the pathway at this level is a conserved feature in the genetic architecture of *Musca* and *Drosophila*.

Keywords *Transformer2* · *Musca domestica* · Sex determination · Splicing regulation

Introduction

Among insect species, a variety of seemingly different cues have been found to determine sex (Nöthiger and Steinmann-Zwicky 1985). In an attempt to understand how and why different cues have evolved, we are conducting a comparative study between the sex-determination pathway of *Drosophila melanogaster* and that of a distant dipteran relative, the housefly *Musca domestica*. In *D. melanogaster*, the ratio of X chromosomes to sets of autosomes (X:A ratio) serves as the primary signal in sex determination (reviewed in Cline and Meyer 1996; Parkhurst and Meneeley 1994; Schutt and Nothiger 2000). This signal defines the state of activity of *Sex-lethal* (*Sxl*), which is the top switch in the pathway. *Sxl* controls all aspects of sexual dimorphic development through a short cascade of subordinate genes. In XX:AA zygotes, *Sxl* is activated and keeps its active state by an autoregulatory function (Bell et al. 1991). Its product, SXL protein, regulates the splicing of the *transformer* gene (*tra*), which results in the production of functional TRA protein (Inoue et al. 1990; Sosnowski et al. 1989). TRA, together with TRA2, a co-factor that is expressed in both sexes, promotes female-specific splicing of the bottom-most element in the pathway, *dsx* (Amrein et al. 1988; Baker and Wolfner 1988; Burtis and Baker 1989; Hoshijima et al. 1991). As a consequence, female-specific *dsx* products (DSX^F) are generated which act as transcriptional regulators to direct female differentiation. In X:AA zygotes, *Sxl* remains inactive and, in the absence of functional SXL products, *tra* pre-mRNA is spliced into the non-functional male mode. When TRA protein is absent, *dsx* is spliced into the male-specific mode and produces mRNAs that encode the male-specific activity (DSX^M) of this gene. The pathway bifurcates downstream of *tra* as courtship behavior is controlled by a different target than *dsx*, namely by *fruitless* (*fru*). In the absence of an active TRA and TRA2 complex, male-specific splice variants of *fru* are produced which are essential for normal male courtship (Ito et al. 1996; Ryner et al. 1996).

The *Drosophila transformer2* gene encodes a protein with an RNA-recognition motif (RRM), flanked by two arginine-rich/serine-rich regions (RS-domains). It is a member of the family of splicing regulator proteins (SR-proteins) that control the utilization of alternative splice sites (Goralski et al. 1989; Manley and Tacke 1996). RS-domains mediate protein-protein interactions to facilitate

Edited by D. Tautz

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the formation of both spliceosomal and regulatory splicing complexes (Amrein et al. 1994; Kohtz et al. 1994; Wu and Maniatis 1993). In *Drosophila* females, TRA2 together with TRA and other RS-proteins build up a splice-enhancing complex that promotes the use of a 3′ splice site upstream of female-specific exon 4 of the *dsx* gene (Lynch and Maniatis 1995; Tian and Maniatis 1993). The TRA/TRA2 complex binds to six nearly identical copies of an exonic splicing enhancer (ESE) and a single purine-rich element (PRE) allowing the assembly of a functional spliceosome at the adjacent 3′ splice site (Lynch and Maniatis 1996; Tian and Maniatis 1993). Additionally, TRA2 is involved in 5′ splice site choice of *fru* pre-mRNA. Similar to *dsx*, this regulation depends on binding to a specific set of ESEs present in the *fru* pre mRNA (Heinrichs et al. 1998; Lam et al. 2003).

In *M. domestica*, sex is determined by the presence or absence of a dominant male-determining factor *M* (Perje 1948). When *M* is present in the zygote, it prevents the activation of the female-promoting *F* gene and thereby imposes male development. *M* is usually located on the Y chromosome, but it can also be found on different autosomes in natural populations (Rubini et al. 1972). In the absence of *M*, *F* is active and directs female development (Dubendorfer et al. 2002). *F* was mapped to chromosome 4 and is genetically defined by two mutant alleles. The gain-of-function allele F^{D} behaves as a dominant female determiner since it cannot be repressed by *M* (McDonald et al. 1978; Rubini et al. 1972). The recessive allele F^{man}, on the other hand, has features of a loss-of-function mutation. F^{man} homozygous individuals develop as males, even in the absence of *M* (Schmidt et al. 1997). To become zygotically active, *F* requires its own maternal activity, suggesting that this gene relies on an autoregulatory function to maintain a female-promoting active state (Dubendorfer and Hediger 1998). Though *F* in many regards behaves as *Sxl* in *Drosophila*, the *Musca* homologue of *Sxl* is an unlikely candidate for *F* because it is equally expressed in both sexes (Meise et al. 1998). It appears that the components at the top of the sex-determining pathway are different in *Drosophila* and *Musca*. However, the *dsx* homologue of *Musca* (*Mddsx*) corresponds not only structurally but also functionally to *Drosophila dsx*, the bottom most regulator in the pathway (Hediger et al. 2004). Furthermore, sex-specific regulation of *Mddsx* is based on a differential splicing mechanism that is very similar to the one described for *Drosophila dsx*. In *Musca* females, an alternative 3′ splice site is utilized in the *Mddsx* pre-mRNA (Hediger et al. 2004). The female-specific exon contains several putative ESEs and a PRE, suggesting that conserved *trans*-acting factors are involved in promoting the use of this female-specific acceptor site. This finding prompted us to isolate the *Musca* homologue of *tra2* since this component of the complex is thought to interact directly with the ESEs and PRE (Lynch and Maniatis 1996). Furthermore, previous characterization of *tra2* homologues in *Drosophila virilis* (*Dvtra2*) and in humans (*tra2*α and *tra2*β) suggested that this gene has been well preserved in structure and function (Amrein et al. 1988; Beil et al. 1997; Chandler et al. 1997; Dauwalder et al. 1996).

We here present the isolation and characterization of the *tra2* homologue of *M. domestica* (*Mdtra2*), and we provide evidence for an essential function of this gene in female development of the housefly, not only in regulating targets of *F* but also in maintaining the female state.

Materials and methods

PCR with degenerate primers

The 5′ primers correspond to sequences located in the middle of the RRM; 3′ primers correspond to sequences in the extended homology region downstream of the RRM. The following primers were used:

forward

MAR25 :

5′ TGY CTI GGN GTN TTY GGS YT R 3′

MAR5 :

5′ MGN TCI CGI GGN TTY TGY TTY R 3′

reverse

MAR17 :

5′ GT RTG IGS ICG YTK NGT DAT NGA 3′

Genomic DNA templates were prepared from adult males and females of a wild-type standard XX/XY strain. A first PCR round was performed with MAR25 and MAR17, followed by a second amplification with MAR5 (nested) and MAR17. We used standard concentrations of Mg and nucleotides. An amount of 50 ng DNA template was amplified in a total volume of 50 µl with 100 pmol each primer. PCR conditions for first amplification were: denaturation at 95°C for 2 min, 35 cycles (denaturation at 95°C for 30 s, annealing at 42°C for 1 min and extension at 72°C for 1 min) and extension at 72°C for 5 min. Second amplification with nested primers involved: denaturation at 95°C for 2 min, 5 cycles (denaturation 95°C for 30 s, annealing at 42°C for 1 min and extension at 72°C for 1 min), then 25 cycles (denaturation 95°C for 30 s, annealing at 62°C for 1 min and extension at 72°C for 1 min) and a final extension at 72°C for 5 min. Fragments from 100 to 700 bp were gel-eluted, some reamplified, using the same conditions as with nested PCR. Subcloning and sequencing of the candidate fragments were carried out by standard procedures.

Rearing of *Musca* strains

Strains were reared as described previously (Schmidt et al. 1997). Since small populations of larvae are difficult to raise on standard medium, larvae obtained from injected embryos were raised on pig dung.

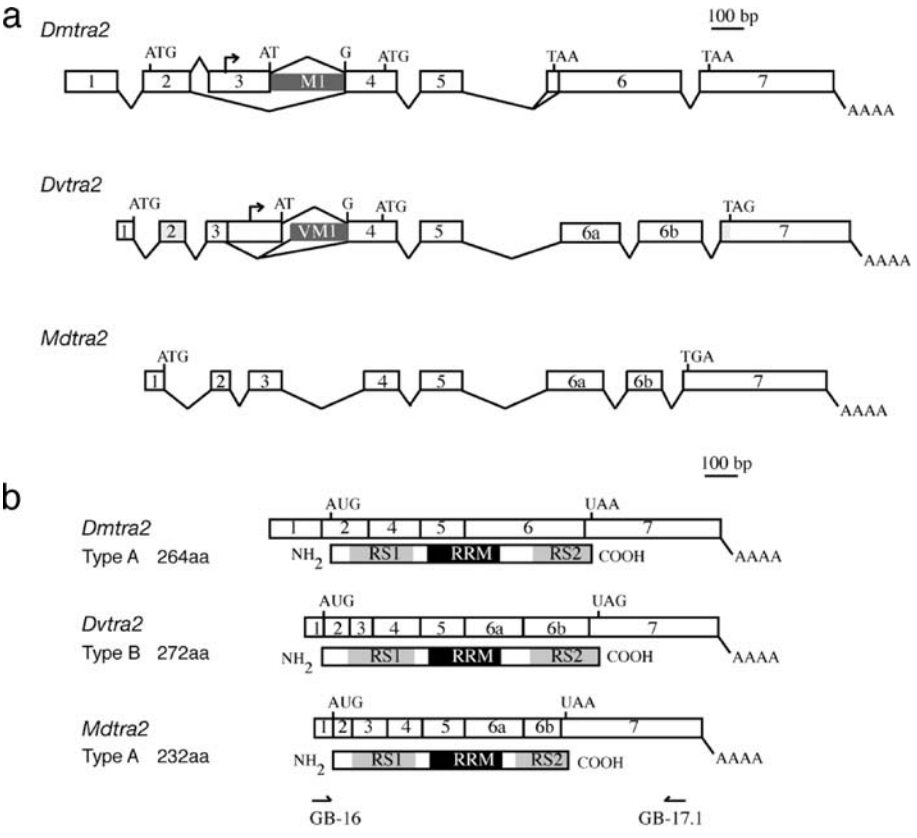
Strains of *M. domestica*

(1) Wild-type strain: females *XX*, males *XY*; (2) autosomal *M* strain: females *XX; pw bwb/pw bwb*, males *XX; $M^{III}pw^{+}bwb^{+}$/pw bwb*; (3) F^{D} strain: females M^{III}/M^{III}; $F^{D}Ba/F^{+}Ba^{+}$, males M^{III}/M^{III}; $F^{+}Ba^{+}/F^{+}Ba^{+}$; (4) F^{man} strain: females *XX;* F^{man}/F^{+}, males *XX;* F^{man}/F^{man} (Schmidt et al. 1997); (5) F^{man} of F^{D} strain: females XX; $F^{D}Ba/F^{man}Ba^{+}$, males $F^{man}Ba^{+}/F^{man}Ba^{+}$.

To obtain a pure female progeny, females of strain 1 were crossed to males of strain 4. Pure male progeny was obtained by crossing females of strain 1 with males of strain 3.

In situ hybridizations

In situ hybridizations were carried out according to the protocol of Tautz and Pfeifle (1989).



d

	RS 1		RS 2	
	Arg	Ser	Arg	Ser
Mdtra2	27%	25%	35%	28%
Dmtra2	26%	32%	33%	18%
Dvtra2	20%	32%	34%	25%

Fig. 1 *tra2* genes in *Drosophila melanogaster*, *D. virilis* and *Musca domestica*. **a** Genomic organization of *Dmtra2*, *Dvtra2* and *Mdtra2*. Testis-specific transcription start sites in *Dmtra2* and *Dvtra2* are marked by *small arrows*. *M1* and *VM1* are alternatively spliced introns. **b** Comparison of the structures of the major splice variant of *Dmtra2* and *Dvtra2* (Chandler et. al. 1997) with the *Mdtra2* transcript and the corresponding protein (*below*). The RNA-recognition motif (*RRM*) is marked in *black* and the arginine-rich/serine-rich (*RS*) domains are in *grey*. **c** RT-PCR amplifications with primers in exon 1 (GB-16 in *b*) and exon 7 (GB-17.1 in *b*) of *Mdtra2* that encompass the complete ORF (*E*♀ female embryos, *E*♂ male embryos, *L*♀ female larvae, *L*♂ male larvae, *A*♀ female adults, *A*♂ male adults, *ov* ovaries, *tes* testes). **d** Percentages of arginine and serine residues present in the RS 1 and RS 2 domains of DmTRA2, DvTRA2 and MdTRA2

Injection of dsRNA and RT-PCR analysis

A cDNA fragment of the *Mdtra2* gene was amplified with primers each flanked by T7 promoter sequences at the 5′ end. The resulting 560-bp fragment extends from 14 bp upstream to 385 bp downstream of the RRM. This template was used to produce dsRNA fragments by in vitro transcription with T7 RNA polymerase. The dsRNA was precipitated with ethanol and resuspended in injection buffer to a final concentration of 1 μg/μl. Embryos were collected within 1 h of egg laying and dechorionated prior to injection (Hediger et al. 2001). Injected embryos were allowed to develop at room temperature.

For transcript detection by RT-PCR, total RNA of single adult flies was extracted according to the RNeasy Mini protocol of Qiagen. RT-PCR reactions were performed using the Titan One Tube RT-PCR Kit (Roche).

Results

Isolation of a *tra2* homologue in *Musca*

A unique structural feature among previously isolated homologues of *tra2* is the presence of an extended stretch of 57 bp of homology downstream of the RRM. We designed degenerate primers that hybridize within the RRM (5′) and within the extended homology block (3′) and used these in various combinations to amplify genomic DNA from a wild-type laboratory strain of *M. domestica*. Two of 35 sequences recovered were identical and displayed the same type of extended RRM homology when compared to *tra2* sequences of *D. melanogaster* (*Dmtra2*) and *D. virilis* (*Dvtra2*). We therefore referred to this sequence as *Mdtra2*. 5′ and 3′ RACEs on poly $(A)^+$ RNA prepared from *Musca* embryos resulted in the assembly of a transcript of 1.4 kb in length (GenBank Accession No AY847518). Genomic fragments covering the 1.4-kb transcribed region were isolated by screening a *Musca* genomic library and by PCR from genomic templates. From aligning genomic and cDNA sequences we deduced that the assembled *Mdtra2* transcript is composed of eight exons (Fig. 1a).

An alignment of genomic and cDNA sequences of *Dmtra2*, *Dvtra2* and *Mdtra2* revealed that at least two introns are located at the exact same position, namely between exons 4 and 5 and between exons 5 and 6 (Fig. 1a). In addition, exon 6 of *Dmtra2* is split by an intron in *Dvtra2* and *Mdtra2* at exactly the same location. We refer to these exons as 6a and 6b (Fig. 1a; Chandler et al. 1997). In *Dmtra2*, the translational start codon is located in exon 2, whereas in *Dvtra2* the last three nucleotides of exon 1 represent the putative start codon for translation (Fig. 1a). Likewise, we found that the upstream-most translational start site in the assembled *Mdtra2* transcript is also located

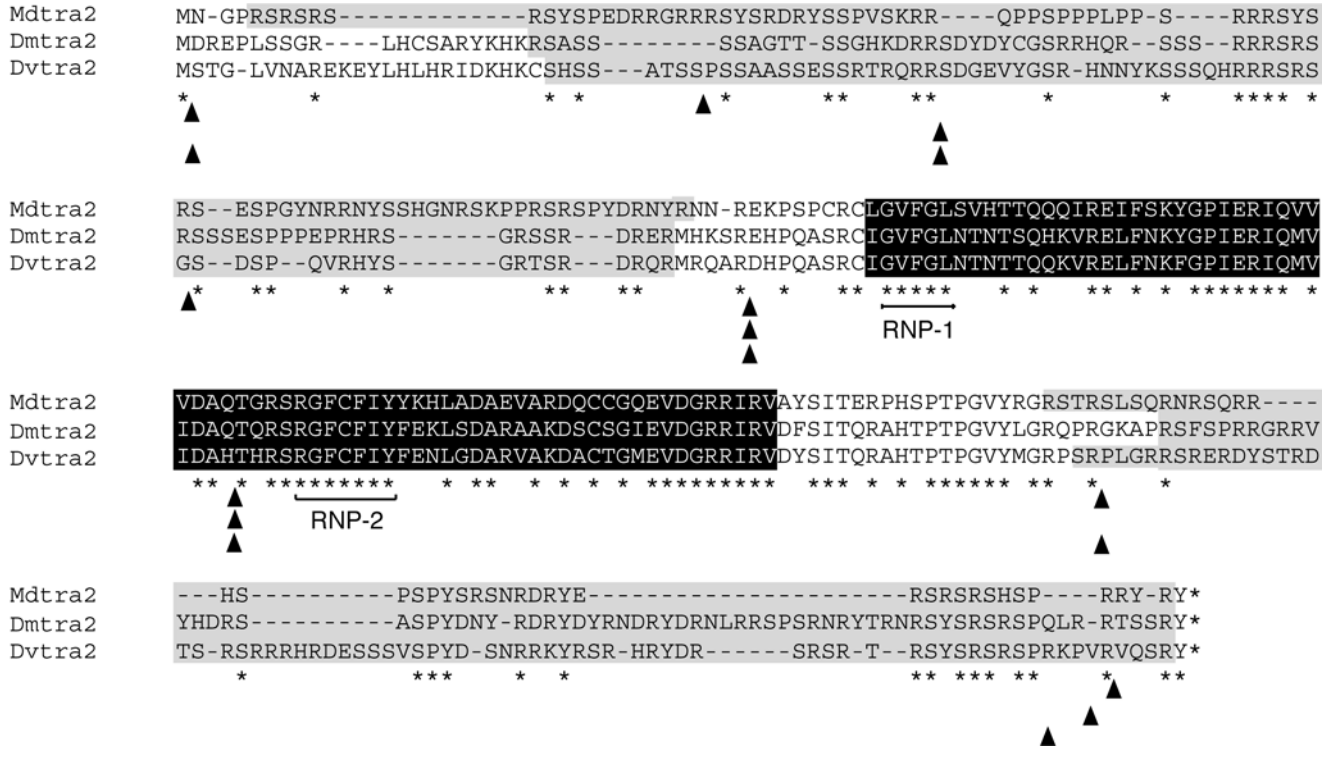


Fig. 2 Sequence alignment of the major protein variants of DmTRA2, DvTRA2 and MdTRA2. The RNA recognition motif (RRM) is in *black*; RS domains are *grey*. *Asterisks* indicate the positions of amino acids that are identical in all three sequences. *Arrowheads* below sequences show the position of introns. Note the high amount of identical amino acids within the RRM, whereas both RS domains are less conserved but abundant in arginine and serine. *RNP-1* and *RNP-2* indicate the positions of two ribonucleoprotein identifier sequences, which are highly conserved between RRM proteins

at the very 3′ end of exon 1. Based on these features *Mdtra2* seems to be structurally more closely related to *Dvtra2* than to *Dmtra2*. Different from *Dvtra2* and *Dmtra2*, however, we did not detect alternatively spliced products in *Mdtra2*. We also found no evidence for the presence of an internal testis-specific promoter (see below). It thus appears that regulation of *Mdtra2* is less complex at the level of transcription and splicing than that of the corresponding gene in *D. virilis* and *D. melanogaster* (Fig. 1a).

Mdtra2 produces a single transcript in soma and germ line of both sexes

In *D. melanogaster*, three distinct TRA2 protein variants ($TRA2^{264}$, $TRA2^{226}$ and $TRA2^{179}$) arise as a result of alternative splicing and the use of different transcription start sites (Amrein et al. 1990; Mattox et al. 1990). In the soma, $TRA2^{264}$ and $TRA2^{226}$ function redundantly to regulate female-specific splicing of *dsx* (Mattox et al. 1996). In the male germline, $TRA2^{226}$ originates from a different transcription start site and is necessary and sufficient for

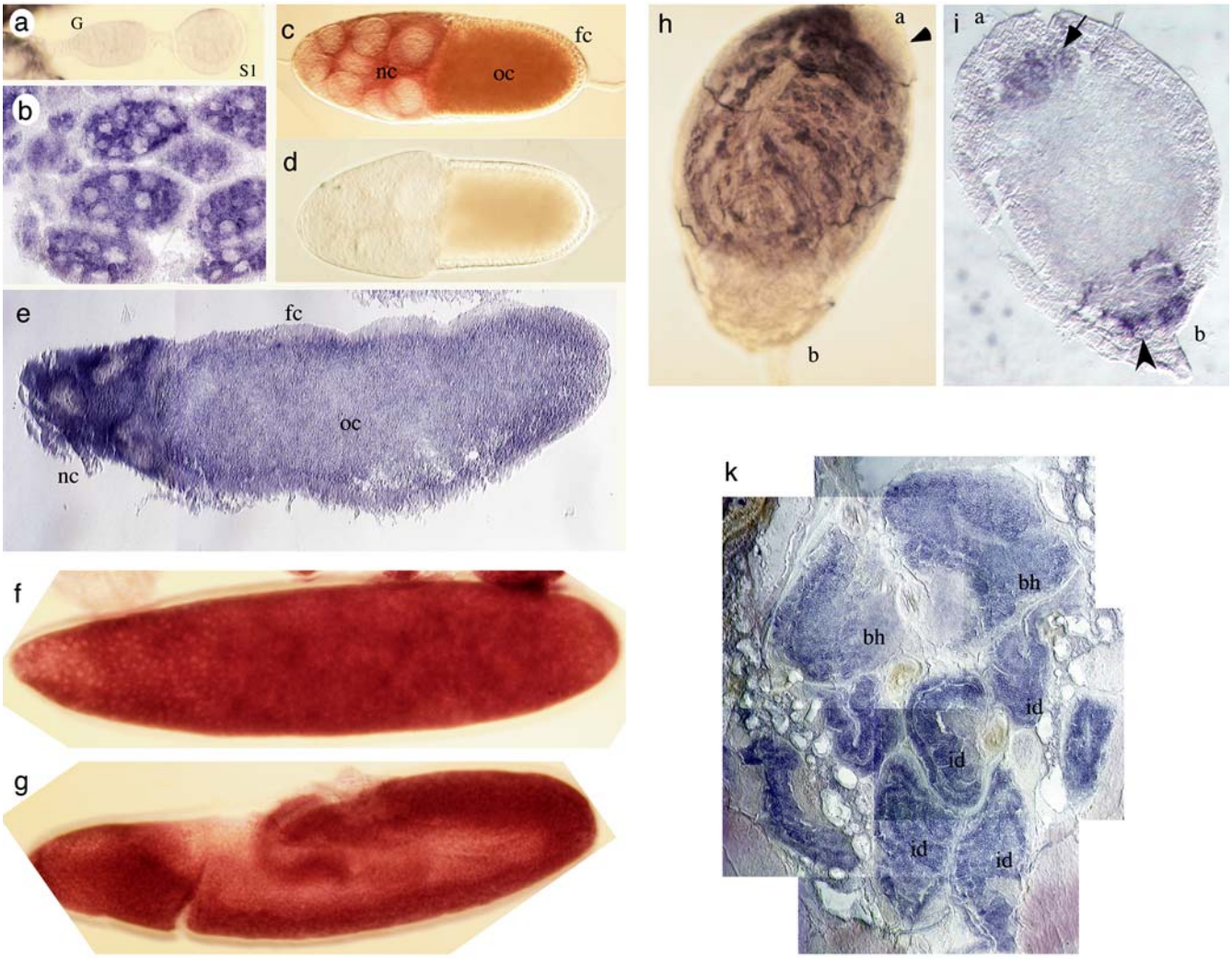


Fig. 3 Distribution of *Mdtra2* transcripts in different tissues of *Musca*. **a**–**e** Localization of transcripts at different stages of oogenesis. **a** *Mdtra2* transcripts are not detected in the germarium (*G*) and in stage 1 cysts (*S1*). **b** *Mdtra2* transcripts first accumulate in nurse cells at stage 4. **c** At stage 7, *Mdtra2* transcripts accumulate in the cytoplasm of nurse cells (*nc*) and in the growing oocyte (*oc*), but are not detected in the somatic follicle cells (*fc*). **d** No signal is observed with a *Mdtra2* sense probe at the same stage 7. **e** Section through a late egg chamber with uniform distribution of *Mdtra2* transcripts in the oocyte (*oc*) and in the degenerating nurse cells (*nc*). **f**, **g** Distribution of *Mdtra2* transcripts in embryos. High levels of uniform staining are found at the blastoderm stage (**f**) and at the germband extension stage (**g**). **h**, **i** Distribution of *Mdtra2* transcripts in adult testes. **h** Whole-mount staining of a testis: transcripts are prominently expressed in differentiating spermatids, but excluded from the area beneath the apical end which contains stem cells and early stages of spermatogenic development (*a*, *arrowhead*). **j** Section through a testis: transcripts are detected in groups of cells at the apical end (*a*) which are presumed to be clusters of primary spermatocytes (*arrow*). At the basal end (*b*), transcripts are found in cells which are presumed to be somatically derived (*arrowhead*). **k** In situ hybridization on section through the anterior part of a third instar larva. High levels of transcripts are detected in cells of the brain hemispheres (*bh*) and in the epithelial cells of the imaginal discs (*id*)

male fertility, ensuring correct processing of *exuperantia* pre-mRNA (Hazelrigg and Tu 1994) and controlling the splicing of its own pre-mRNA (Mattox et al. 1990).

In *Mdtra2* only a single transcript of about 1.4 kb in size was detected in male and female embryos by northern blot analysis (not shown). Using a pair of primers in exons 1 and 7 (Fig. 1b), we tested cDNA samples prepared from different tissues and stages for the presence of alternatively spliced products. A single amplification product was recovered in all cases (Fig. 1c). RT-PCR amplification with primers located at different positions in the intron between exon 3 and 4, which corresponds to M1 in *Dmtra2* and VM1 in *Dvtra2*, did not yield any products in poly $(A)^+$ samples of dissected testes. These results argue against the presence of male germline-specific transcripts in *Mdtra2*. Rather they suggest that *Mdtra2* is expressed as a single transcript of 1.4 kb in all tissues. The longest ORF of this transcript encodes a protein of 232 aa and shows a best fit with the $DmTRA2^{264}$ and $DvTRA2^{272}$ isoforms (Fig. 1b).

Alignment of $MdTRA2^{232}$ protein sequence with $DmTRA2^{264}$ shows an overall similarity of 57%, and of 87% within the RRM. Two regions of low-complexity, RS 1 and RS 2 which are rich in arginine and serine, flank the RRM (Fig. 2). Most identities in these regions consist of repeated Arg/Ser residues (Fig. 1d). Most importantly, the predicted MdTRA2 protein contains a highly conserved linker region between the RRM and the RS 2 domain (76% similarity). This structural feature appears to be specific to *tra2* homologues within the class of RRM-containing genes.

Northern blot analysis and RT-PCR experiments indicated that *Mdtra2* is transcribed continuously throughout the life cycle of the housefly. However, transcripts are more abundant in females and early embryos (data not shown), suggesting that there is a maternal deposit in embryos. This was tested by in situ hybridization with a DIG-labeled *Mdtra2* RNA probe. We confirmed that levels of transcripts accumulate in previtellogenic and late stages of oogenesis and in early embryos (Fig. 3a–g). The uniform expression pattern gradually declines during embryonic development. At late larval stages, we observe an accumulation of *Mdtra2* transcripts specifically in cells of imaginal tissues and the CNS (Fig. 3k). In testes of the adult male, transcripts are prominently located in the compartment containing differentiating germ cells (Fig. 3h). Notably, transcripts are absent in the most apical region where germline stem cells and early dividing spermatogonial cells reside (arrow in Fig. 3h).

Compromising *Mdtra2* activity causes female-to-male sex reversal

In *D. melanogaster*, *tra2* acts as an indispensable cofactor of TRA in the female-specific splicing of *dsx* (Tian and Maniatis 1993). Animals with a female XX genotype are sex reverted and develop into sterile males when mutant for *tra2* (Amrein et al. 1990). We tested the function of *Mdtra2* in *Musca* by applying RNAi (McGregor et al. 2001). We used a 384-bp cDNA fragment derived from an embryonic *Mdtra2* cDNA clone to synthesize *Mdtra2* dsRNA. This fragment includes the RRM, the linker region and part of the RS 2 domain and was injected into pre-blastoderm stage embryos of the autosomal *M* strain (strain 2, see Materials and methods) which allowed us to distinguish between genotypically female and male animals. The male-determining factor *M* on chromosome 3 is linked to the dominant wild-type alleles of *bwb* (*brown body*) and *pw* (*pointed wings*) whereas the non- *M* carrying chromosome 3 is marked with mutant alleles of these loci. Since crossing-over is virtually absent in male flies, these phenotypical markers are reliable indicators for presence or absence of *M*, i.e., for the genetic sex: males ($M\ pw^+bwb^+/pw\ bwb$) have a wild-type body color and normal shaped wings, whereas females (*pw bwb/pw bwb*) are brown and have pointed wings.

Of about 800 injected embryos, 34 individuals heterozygous and 35 homozygous for *bwb* and *pw* survived to adulthood. All 34 heterozygous flies (male genotype) had a normal male phenotype (Table 1). Single fertile flies of this type when crossed to untreated *bw pw* females produced offspring with a normal 1:1 sex ratio, confirming the presence and transmission of a functional *M* factor. Of the 35 *bwb pw* mutant survivors (female genotype), 20% displayed male-like external genitalia and eye-distance (Fig. 4g, h). Of the *bwb pw* individuals 68% were intersexual composed of a mixture of male and female structures (Fig. 4e, f) and only 11% developed into normal-looking

Table 1 Sexual phenotypes of *Mdtra2* dsRNA injectees (*N.D* not determined)

Genotype	Percentage (N^a)	Interocular width	Genitalia	Gonads	Sex of progeny[b]
bwb pw/bwb pw (female)	20(7)	♂	♂	Testes	♀ only
	46(16)	♀	♂	Testes	♀ only
	11(4)	♀	ix[c]	N.D.	
	3(1)	♂	ix	N.D.	
	8(3)	♂	♀	Ovaries	
	11(4)	♀	♀	Ovaries	
+ + M/*bwb pw* (male)	100(34)	♂	♂	Testes	♀ and ♂
XY	42(30)	♂	♂	Testes	♀ and ♂
XX	18(13)	♂	♂	Testes	♀ only
XX	37(27)	♀	♂	N.D.	N.D.
XX	3(2)	♀	♀	N.D.	

[a] *N* Total number of flies examined

[b] Flies with male phenotype were tested for presence of *M* by crossing to wild-type females (with *M* mixed progeny, without *M* female only progeny)

[c] *ix* Intersexual composed of male and female structures

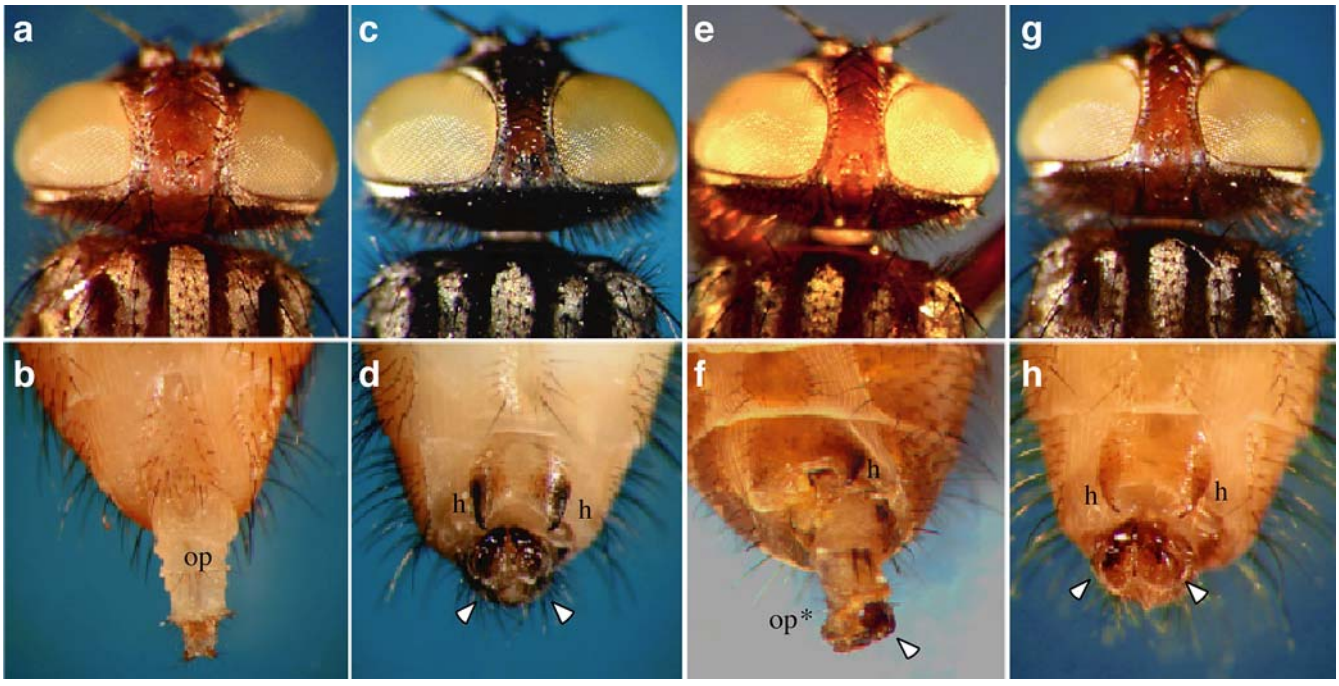


Fig. 4 Sex reversion caused by injections of *Mdtra2* dsRNA into embryos. The male determining *M* factor is autosomal and linked to *brown body*$^{+}$ (*bwb*$^{+}$). Genotypic males have a normal black body pigmentation. Females are *bwb/bwb* and have a brownish body color. Phenotypically, male heads can be distinguished from female heads by a significantly narrower interocular distance. The external female genitalia are characterized by the presence of an ovipositor (*op*), whereas males have a darkly pigmented copulatory apparatus and exhibit characteristic horn-like structures (*h*) at the tip of sternite 5. **a** Head of a non-injected female (*bwb/bwb*). **b** Genital region of the same female with a stretched ovipostitor (*op*). **c** Head of a non-injected male (*M bwb*$^{+}$/*bwb*). **d** The genital region which display a pigmented copulatory apparatus (*white arrowheads*) and two horn-like structures at the tip of sternite five (*h*). **e** Head of an injected genotypically female displaying a male interocular distance. **f** Genitalia of the same female are composed of a partial ovipositor (*op**) covered with male-like tissue (*white arrowhead*) and a horn-like structure (*h*) on sternite 5. **g**, **h** An RNAi-treated individual with a female genotype and a normal male morphology of the head and a male-like genital region

fertile females. In single crosses with untreated *bwb pw* females, all male-looking *bwb pw* flies and even 62% of the intersexes with male genitalia produced offspring (Table 1). All crosses yielded exclusively female progeny. This result clearly demonstrates that these *bw pw* males and intersexes did not carry an *M* and therefore had a female genotype.

The masculinizing effect of *Mdtra2* dsRNA was also confirmed in an unmarked standard XX/XY strain. After injections in embryos of this strain, we again recovered a high percentage of intersexual flies and phenotypic males. Only 3% displayed a normal female morphology. To test for the presence of *M*, which is located on the Y chromosome in this strain, we crossed the male flies to untreated XX females. Thirteen of 43 phenotypic males produced only daughters indicating that they had a female XX genotype (Table 1). On the other hand, no phenotypic abnormalities were observed in genotypically male flies that were injected with *Mdtra2* dsRNA. We thus conclude that *Mdtra2* is essential for female development.

Mdtra2 is required for female splicing of *Mddsx*

In *D. melanogaster*, TRA2 is required for female-specific processing of *dsx* pre-mRNA. Given the similar mechanism by which *Mddsx* is regulated in the housefly (Fig. 5A), we tested for presence of female-specific and male-specific *Mddsx* mRNAs in animals in which *Mdtra2* was silenced by RNAi. In untreated *bw pw* mutant females, only female-specific messages of *Mddsx* are detectable (Fig. 5B). Upon injections of *Mdtra2* dsRNA, however, the sex-reverted *bwb pw* flies contain not only female-specific messages, but also a substantial amount of male-specific *Mddsx* mRNA (Fig. 5B). These male-specific transcripts must have arisen from a failure to effectively utilize the splice acceptor site of the female-specific exon. This situation compares to that in *Drosophila* XX animals with impaired *tra2* function (Fortier and Belote 2000). Sex reversion of houseflies with compromised *Mdtra2* activity can thus at least in part be explained by a failure in female processing of *Mddsx*. The degree of sexual transformation in these flies is much stronger than that observed in flies injected with *Mddsx* dsRNA where only the gonads were affected (Hediger et al. 2004). This suggested to us that *Mddsx* is not the only target of *Mdtra2* in the sex determination pathway.

Mdtra2 is a positive regulator of *F*

F has been proposed to be an upstream regulator of *Mddsx* (Hediger et al. 2004). It is conceivable that, like *tra* and *tra2*

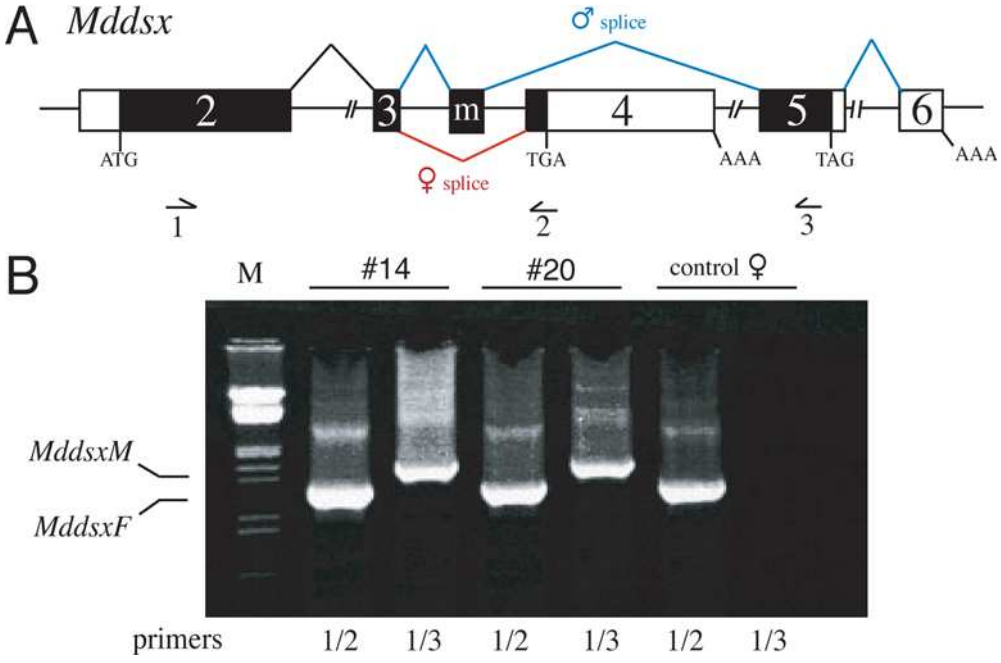


Fig. 5 *Mdtra2* is required for female splicing of *Mddsx*. **A** Genomic organization of *Mddsx* (Hediger et al. 2004); male-specific splice pattern is indicated in *blue* and the female-specific splice pattern in *red*. *Arrows* mark the positions of primers used below. **B** RT-PCR amplifications with male-specific set (*1/3*) and female-specific set (*1/2*) of *Mddsx* primers. RNA preparations from a control adult female (non-injected *bwb/bwb* animal) produced only a product with the female-specific set (1/2). RNAi-treated *bwb/bwb* individuals: fly#14 had a male outer morphology, fly#20 displayed a male genital region and a female interocular distance. Preparations of RNA from both adult flies yielded a substantial amount of male-specific products

in *Drosophila*, *F* and *Mdtra2* act cooperatively to set *Mddsx* into the female mode. To determine the level at which *Mdtra2* acts relative to *F*, we silenced *Mdtra2* in a strain carrying a gain-of-function allele of *F*, F^{D}. Males of this strain are homozygous for F^{+} and *M* (*M*/*M*; F^{+}/F^{+}). Zygotes that carry one copy of F^{D} develop into fertile females even in the presence of two *M*s (*M*/*M*; F^{D}/F^{+}). The F^{D} allele in this strain is tightly linked to the dominant marker *Ba* (*Bald abdomen*). We injected the same dsRNA fragments of *Mdtra2* into 500 embryos of which 71 survived to adulthood; 33 of these were *Ba* (*M*/*M*; F^{D}/F^{+}) and developed into normal-looking females; 38 were Ba^{+} and were males (*M*/*M*; F^{+}/F^{+}). Different from the previous silencing experiments, we could not observe any male structures in the dimorphic regions of the genotypically female class of survivors. Most of these females, however, were sterile and laid no eggs. Upon examination of their gonads, we observed that only 2 of 24 contained normal ovaries with mature oocytes, whereas the remaining had ovotestes (Fig. 6e). These ovotestes were much smaller in size and typically contained an apical cap of pigmented testicular-like sheath. Shape and morphology of the ovotestes were similar to those observed in flies with compromised *Mddsx* activity (Hediger et al. 2004). We found spermatogenic stages beneath the pigmented sheath (Fig. 6f). As the sexual fate of germ cells in *Musca* is solely dictated by the sex of the surrounding gonadal tissue, the occurrence of male differentiated germ cells is a strong indication that the overlying gonadal soma was transformed into testicular tissues (Hilfiker-Kleiner et al. 1994).

In contrast to the autosomal *M* and XX/XY strains used in the previous experiments, the F^{D} strain appeared to be resistant to the masculinizing effect of *Mdtra2* RNAi in non-gonadal soma. We infer from this result that, although the wild-type allele of *F* requires functional *Mdtra2* to maintain its female-promoting activity, the dominant F^{D} allele does not depend on *Mdtra2* to direct female differentiation in non-gonadal soma. This conclusion places *Mdtra2* as a positive regulator upstream of *F*. On the other hand, the masculinized gonadal phenotypes in F^{D} females with compromised *Mdtra2* activity suggest that *Mdtra2*, at least in this tissue, acts downstream or in parallel to *F*.

Suppression of male courtship requires *Mdtra2*

The allele F^{man} has been described as a hypomorph of *F* (Schmidt et al. 1997). Flies homozygous for this allele and with no *M* develop into males but 40% of these males produce no progeny. This sterility is not due to defects in spermatogenesis or abnormal gonadal or genital development, but to poor performance of these males to engage in courtship and copulation (S. Kaeppeli, unpublished results). This behavioral defect is aggravated when F^{man} homozygous males derive from a mother that carries a F^{D} allele. All of her sons are behaviorally sterile as they make no attempts to copulate. A likely explanation is that courtship behavior of these males is suppressed by a residual female-promoting activity of F^{man} the level of which depends on maternal activity of *F*. Consistent with this presumption is the finding that the introduction of an *M* factor into F^{man} homozygous males renders them fertile.

If male courtship behavior is suppressed by *F*, it is conceivable that this process also requires functional *Mdtra2*. We thus injected *Mdtra2* dsRNA into embryos derived from a cross between F^{D}/F^{man} mothers (strain 5) and F^{man}/F^{man} fathers (strain 4). The surviving F^{man}/F^{man} males were tested for fertility in single matings with XX wild-type females. Twenty-one of 31 tested males (68%) sired progeny and thus were apparently fully rescued in

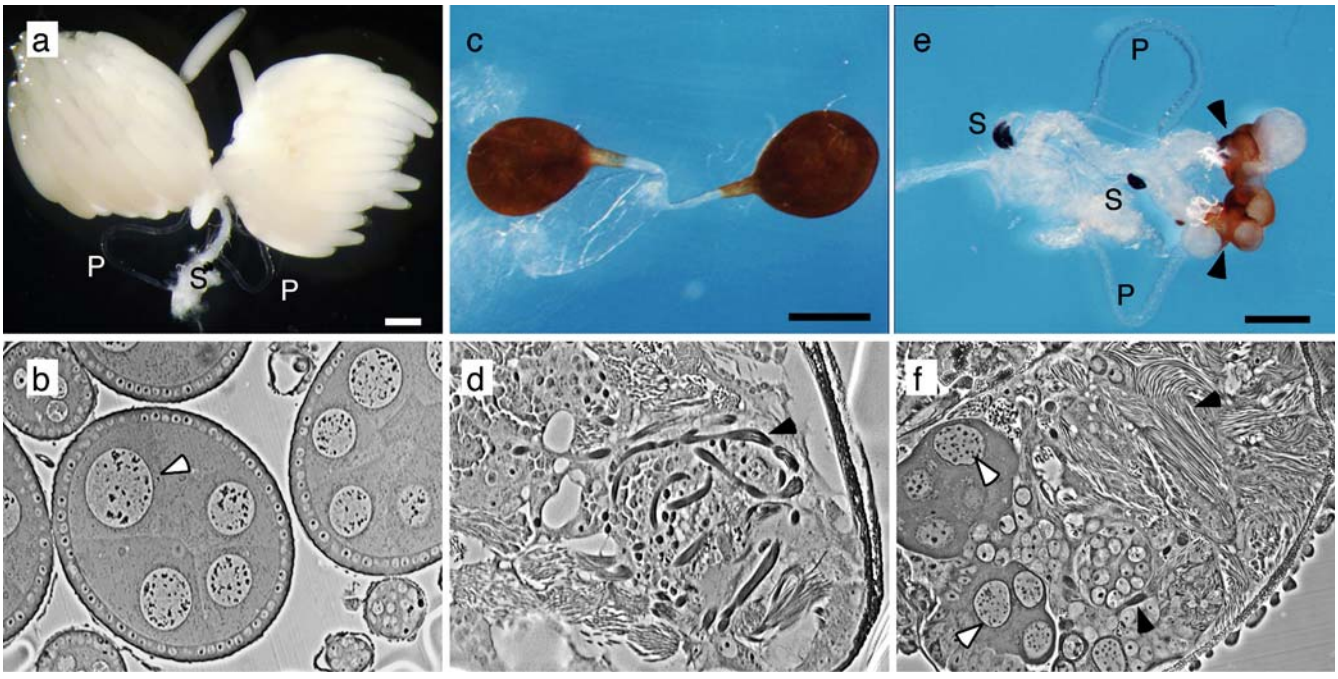


Fig. 6 *Mdtra2* is required for female differentiation of the gonads. **a** Ovaries from a non-injected F^{D}/F^{+} female with fully differentiated eggs (*P* parovaria, *S* spermathecae). **b** Section through the ovary displays several cysts containing polyploid nuclei of nurse cells (*white arrowhead*). **c** Whole-mount of a pair of testes from a male of the same strain. Testes are pear-shaped and covered by a darkly pigmented epithelial sheath. **d** In a section through the testis, bundles of elongating spermatids can be recognized (*arrowhead*). **e** Ovotestes of a F^{D}/F^{+} female treated with *Mdtra2* RNAi. Some female structures (parovaria and spermathecae) are still present, but the gonads are greatly reduced in size and covered by a darkly pigmented male-like epithelial sheath. **f** Microscopic section through the ovotestis reveals the presence of both nurse cell-like nuclei (*white arrowheads*) and elongated bundles of spermatids (*back arrowheads*)

their capacity to copulate with females. This number is only mildly lower than the average 80% of successful matings of injected control wild-type males in single crosses. We conclude that repression of courtship behavior in F^{man} homozygous males requires the activity of *Mdtra2*. This result suggests that, besides an essential role in female differentiation, *Mdtra2* is also engaged in the control of sex-specific behavior to fully implement the female program of development.

Discussion

Mdtra2 is essential for female development in *Musca*

In *Drosophila*, the *tra2* gene plays an important role in female development. It assists the *tra* gene in setting downstream regulators of the sex determination pathway into the female mode. Two direct targets, *dsx* and *fru*, are known, and their regulation by *tra* and *tra2* has been studied in detail (Hedley and Maniatis 1991; Heinrichs et al. 1998; Hertel et al. 1996; Lam et al. 2003; Ryner and Baker 1991; Tian and Maniatis 1993). A structural and functional homologue of one of these targets in *Musca*, *Mddsx*, is sex-specifically regulated in a mode very similar to that of *dsx* in *Drosophila*. We examined whether the *trans*-acting factors involved in this process are conserved in the housefly. We describe the isolation and characterization of a *tra2* homologue in *Musca*. Like its counterpart in *Drosophila*, it is needed for female-specific processing of *Mddsx*. Whether *Mdtra2* participates directly or indirectly in this process remains to be examined, but the presence of well conserved TRA/TRA2 binding sites in the female-specific exon of *Mddsx* pre-mRNA favors a direct participation (Hediger et al. 2004). In *Drosophila*, these sequences serve as binding sites for the TRA/TRA2 splice-enhancer complex. It is thus conceivable that recognition of the female-specific 3′ splice in *Mddsx* also requires the assistance of a MdTRA/TRA2 splice-enhancer complex that binds to these sites. The structural features of MdTRA2 are consistent with such a mode of operation. It contains a well-conserved RRM which is likely to bind to the same type of ESE, as well as RS domains which provide a potential surface for interaction with other RS-containing proteins.

Since *Mdtra2* is equally expressed in females and males, this gene is an unlikely candidate for being the discriminatory component in the complex that determines the female fate. Rather its mode of action may correspond to that of *Drosophila tra2*, namely to act as an essential co-factor in the complex. A more likely candidate for the discriminatory factor in the complex is the product of the *F* gene. We have previously shown that *F* acts upstream of *Mddsx* (Hediger et al. 2004), since *Mddsx* is spliced in the female mode only when *F* is active. If *Mddsx* is a direct target, *F* would functionally correspond to *tra* in *Drosophila*.

A dual role for *Mdtra2* in female development

Activation of the *F* gene in the zygote depends on its own maternally provided activity (Dubendorfer and Hediger 1998). Once *F* is activated, it must be continuously active to keep the cells on the female pathway; removal of its activity at later stages of development leads to male development (Hilfiker-Kleiner et al. 1993). Based on these findings it has been proposed that *F* relies on a feedback mechanism to maintain its female-promoting activity (Dubendorfer et al. 2002). As for the regulation of *Mddsx*, *Mdtra2* may be an essential co-factor for the proposed auto-regulatory function of *F*. A maternal contribution of *Mdtra2* to the activation of *F* in the zygote is suggested by the large amounts of *Mdtra2* transcripts which are deposited into the egg chamber during oocyte maturation. In our model, this initial supply of *Mdtra2* along with maternal *F* is needed to activate zygotic *F* (Fig. 7a). The female-promoting activity by *F* is then sustained by a feedback loop which serves as a cellular memory for the proper execution of the female program. In the male zygote, establishment of this loop is prevented by the action of the paternally transmitted *M* factor and, as a result, male development follows (Fig. 7b). Silencing of *Mdtra2* by RNAi mimics the loop-breaking effect of *M* and results in complete masculinization. We explain this by the loss of *Mdtra2* activity to form an active complex with maternal *F* that activates zygotic *F*. Consequently, the feedback loop of *F* cannot be engaged, and *F* remains functionally off.

The finding that the female-promoting activity of F^D is neither repressed by *M* nor by *Mdtra2* silencing suggests that the F^D allele does not depend on a feedback mechanism to produce active *F* products (Fig. 7c). Rather, the gain-of-function nature of this allele seems to be based on constitutive expression of *F* activity independently of *Mdtra2* or its own maternal activity (Dübendorfer and Hediger 1998). The gonadal soma in these F^D females, however, is affected and becomes transformed into testes. The same phenotype can also be observed in standard XX individuals when *Mddsx* is downregulated by RNAi, suggesting that male transformation of gonadal soma in F^D females with compromised *Mdtra2* activity is caused by misregulation of *Mddsx*. This leads to the conclusion that *Mdtra2* has two genetically separable functions: (1) upstream of *F* as a co-factor of the autocatalytic activity of *F*, and (2) parallel to *F* as a co-factor for the regulation of downstream targets such as *Mddsx* (Fig. 7).

A very similar situation was described in the Mediterranean fruitfly, *Ceratitis capitata*. Pane et al. (2002) reported that the *tra* homologue in the Medfly plays a key role in sex determination based on an autoregulatory function that safeguards the propagation of the female determined state. The *Ceratitis tra* gene is regulated at the level of differential splicing and contains multiple TRA/TRA2 binding sites. The authors propose that the SR protein produced by *Cctra* imposes the female splicing mode on its own pre-mRNA and that of its target, the *dsx* homologue *Ccdsx*. Though the contribution of a putative *Ceratitis* homologue of *tra2* in this process has not yet been studied, this modus operandi would be consistent with our model. It predicts that *F*, which functionally corresponds to *Cctra*, is directly regulated at the post-transcriptional level by its own product

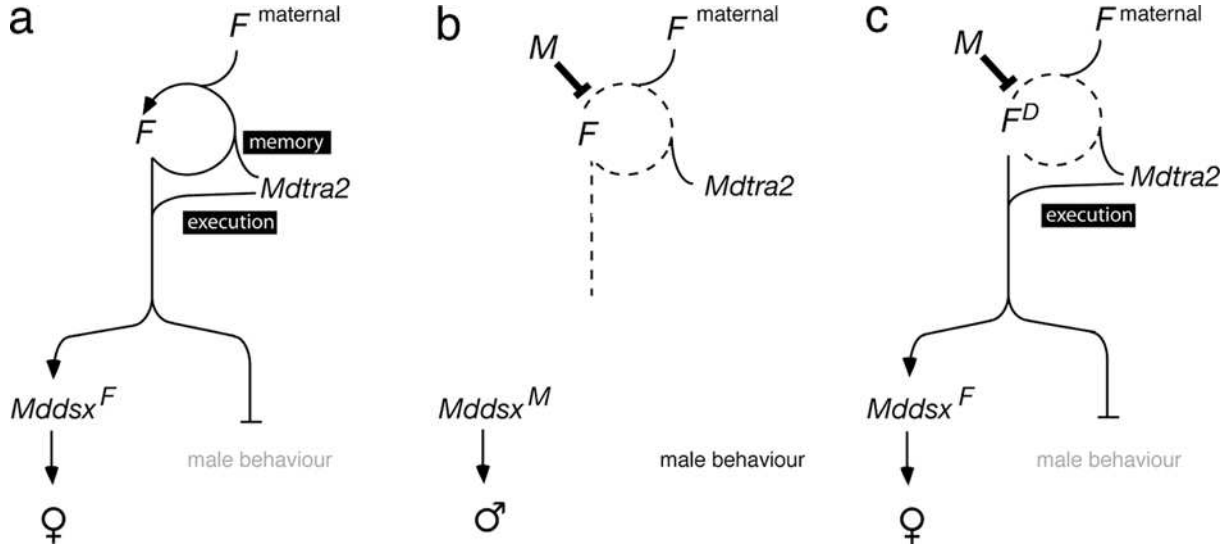


Fig. 7 Model for *Mdtra2* in female development. **a** In the female zygote, *Mdtra2* is required for autoregulation of the female-determining factor *F*. This autocatalytic loop of *F* is established early by maternally provided *F* product and by a maternal deposit of *Mdtra2*, and it is required to maintain the active female-determining state of *F*. Additionally, *Mdtra2* is involved in the execution of the female pathway by assisting *F* in female-specific splicing of *Mddsx* and repressing male courtship behavior via a yet unknown target. **b** In the male zygote, the presence of a paternally transmitted male-determining factor *M* factor prevents the establishment of the loop and *F* will remain in an inactive state. As a result, *Mddsx* will produce a male-specific activity that directs male differentiation and the branch that instates male behavior is derepressed. **c** The gain-of-function F^D allele does not depend on the feedback loop to produce a female-promoting activity. Therefore, *F* and its co-factor *Mdtra2* are dispensable in the activating process. *M* cannot prevent the execution of the female pathway in F^D females, because it exclusively interferes with the activation process of *F*, but not with the downstream functions of *F*

and by *Mdtra2*. In this regard the sex-determining cascades of these two dipteran species appear strikingly similar. It supports the idea that *Drosophila* may be the exception in involving another level of control upstream of *tra* to memorize and execute the instruction given by the primary signal (Saccone et al. 2002). Though well-conserved homologues of *Sxl* are present and expressed in the housefly and in the Mediterranean fruitfly, they appear not to play a role in sexual development (Meise et al. 1998; Saccone et al. 1998).

Mdtra2 and courtship behavior

Besides the regulation of *Mddsx* and *F*, our study implicates *Mdtra2* in the control of male courtship behavior. Our data suggest that *Mdtra2*, together with *F*, represses male courtship behavior. This type of control does not seem to be mediated by *Mddsx*, but by a different branch of the cascade. First, the hypomorphic activity of the F^{man} mutation impairs courtship behavior, but does not affect cuticular differentiation of homozygous males and, second, the splicing pattern of *Mddsx* is male-specific in F^{man} males (Hediger et al. 2004). In *Drosophila*, male courtship behavior is under the control of *tra* but not of *dsx*. Instead, TRA in concert with TRA2 regulates splicing of a different target, namely of *fru*, a gene that is expressed in about 0.5% of the neurons of the central nervous system (Hall 1994; Ryner et al. 1996; Taylor et al. 1994). By a splice enhancer-dependent mechanism, the TRA/TRA2 complex activates a female-specific *fru* 5′ splice site and thereby prevents the production of male-specific *fru* transcripts which are essential for male courtship behavior (Lam et al. 2003). It is conceivable that a functional counterpart to *fru* exists in *Musca* that is regulated by *F* and *Mdtra2*. Likewise, this target may produce a male-specific activity that is essential for courtship behavior. The disturbed sexual behavior of F^{man}/F^{man} males may be caused by some residual activity of *F* which is still sufficient to repress the male mode of this CNS-specific branch of the cascade.

Acknowledgements We are deeply indebted to Dr. Rolf Nöthiger for his continuing support and for many stimulating discussions. We also thank him and Dr. Mary Bownes for helpful comments on this manuscript. Mark Robertson and Dr. Peter Atkinson (Department of Entomology, University of California, Riverside, USA) are gratefully acknowledged for providing degenerate primers and cloning strategies. We thank Simone Kaeppeli for sharing unpublished results. We thank Claudia Brunner for technical assistance and Johanna Nägeli and Raymond Grunder for *Musca* stock keeping. This work was supported by a grant of the Swiss National Foundation (31-67993.02).

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